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(54) Title: METHOD FOR INCREASING BLOOD-BRAIN BARRIER PERMEABILITY

(57) Abstract

The present invention pertains to a method for increasing the permeability of the blood-brain barrier of a host to a molecule present in the host's bloodstream. The method comprises intravenous co-administration to a host of an effective amount of a bradykinin agonist of blood-brain barrier permeability.

METHOD FOR INCREASING BLOOD-BRAIN
BARRIER PERMEABILITY

Background

As our understanding of the nervous system and its
related disorders increases, a wider range of therapeutic
and diagnostic agents will become available. Once these
agents have been identified, it will be necessary to
deliver them to sites of diseased tissue in the central
nervous system. Unfortunately, the existence of the
blood-brain barrier limits the free passage of many types
of molecules from the blood to cells of the central
nervous system.

The physiological basis for the blood-brain barrier
is the brain capillaries, which are comprised of endo-
thelial cells (Goldstein, et al., Scientific American,
255: 74-83 (1986); Pardridge, W.M., Endocrin. Rev., 7:
314-330 (1986)). These endothelial cells are different
from those found in other tissues of the body. In
particular, they form complex tight junctions between
themselves. The actual blood-brain barrier is formed by
these high-resistance tight intercellular junctions which
form a continuous wall against the passive movement of
many molecules from the blood to the brain. These cells
are also different in that they have few pinocytotic
vesicles, which in other tissues allow somewhat un-
selective transport across the capillary wall. In
addition, continuous gaps or channels running through the
cells, which would allow unrestrained passage, are
absent.

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One function of the blood-brain barrier is to protect the brain from fluctuations in blood chemistry. However, this isolation of the brain from the bloodstream is not complete. There does exist an exchange of nutrients and waste products. The presence of specific transport systems within the capillary endothelial cells assures that the brain receives, in a controlled manner, all of the compounds required for normal growth and function. The obstacle presented by the blood-brain barrier is that, in the process of protecting the brain, it excludes many potentially useful therapeutic and diagnostic agents.

There are several techniques that either physically break through the blood-brain barrier or circumvent it to deliver therapeutic or diagnostic agents. Among these are intrathecal injections, surgical implants, and osmotic techniques.

Intrathecal injection allows administration of agents directly into the brain ventricles and spinal fluid by puncturing the membranes surrounding the brain. Sustained delivery of agents directly into the spinal fluid can be attained by the use of infusion pumps that are implanted surgically. These spinal fluid delivery techniques are used to treat brain cancers, infections, inflammation and pain. However, they often do not result in deep penetration of substances into the brain.

Clinicians prefer to avoid intrathecal injections because they frequently are ineffective and can be dangerous. Substances injected intrathecally are distributed unevenly, slowly and incompletely in the brain.

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Since the volume of the spinal fluid is small, increases
in intracerebral pressure can occur with repeated in-
jections. Furthermore, improper needle or catheter
placement can result in seizure, bleeding, encephalitis
and a variety of other severe side effects.

An osmotic approach has been used by Dr. Edward
Neuwelt at the University of Oregon to deliver chemo-
therapeutics and imaging antibodies to tumors in the
brain. (Neuwelt, E.A., Implication of the Blood-Brain
Barrier and its Manipulation, Vols 1 & 2, Plenum Press,
N.Y. (1989)) This technique involves an arterial
injection of a bolus of a hypertonic mannitol solution.
The osmotic differential exerted by the mannitol causes
the endothelial cells forming the barrier to shrink,
opening gaps between them for a brief period. During
this period, the drug is administered into the arterial
system and is carried directly into the brain. The
osmotic approach demonstrates that once past the barrier,
therapeutic agents can be effectively distributed
throughout the brain.

Because of the many risks involved, a 24- to 48-
hour period in an intensive care unit is necessary
following osmotic treatment. Mannitol can cause perma-
nent damage (including blindness) to the eye. If the
barrier is permeable for too long, brain edema results.
Cells of the brain also can be damaged when neurotoxic
substances in the blood, not generally accessible to the
brain, are able to cross the barrier. Finally, there is
a significant incidence of seizures in patients during
and after the procedure.

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Summary of the Invention

The present invention pertains to a method of increasing the permeability of the blood-brain barrier of a host to a molecule contained in the host's bloodstream. This method comprises intravenous co-administration to the host of an effective amount of a bradykinin agonist of blood-brain barrier permeability. The molecule to be delivered to the brain can be either an endogenous molecule or an exogenous molecule that is co-administered sequentially or simultaneously with the bradykinin agonist of blood-brain barrier permeability.

An advantage of the present invention is that it provides a practical means of increasing the permeability of the blood-brain barrier by the intravenous administration of a bradykinin agonist of blood-brain barrier permeability while co-administering a molecule of therapeutic, prophylactic or diagnostic value. In contrast to osmotic treatment or intrathecal delivery, intravenous injection of a bradykinin agonist of blood-brain barrier permeability is significantly less traumatic, does not require surgery and is unlikely to necessitate anesthesia. Furthermore, in contrast to the osmotic techniques which allow entry into the brain of a large variety of molecules present in the blood, including protein, the bradykinin agonist of blood-brain barrier permeability preferentially induces passage through the blood-brain barrier of small molecules.

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The intravenous route of administration of a
bradykinin agonist of blood-brain barrier permeability
offers a number of significant advantages over other
routes of administration (subcutaneous or intramuscular
injection, as well as the more drastic measures of
intrathecal or carotid artery injection). For example,
the intravenous route of administration affords a more
convenient method for providing rapid drug action. The
intravenous route also offers better control over the
rate of administration of drugs; prolonged action can be
provided by administering an infusion either inter-
mittently or over a prolonged period of time. Also, slow
intravenous administration of a drug permits termination
of the infusion if sensitivity occurs. In addition,
certain drugs, because of their irritating properties,
cause pain and trauma when given by the intramuscular or
subcutaneous route and must be given intravenously.
Additionally, some drugs cannot be absorbed by any other
route or are unstable in the presence of gastric juices.
The intravenous route also affords a means of adminis-
tration for the patient who cannot tolerate fluids and
drugs by the gastrointestinal route. Delivering thera-
peutic and diagnostic agents into the brain by an intra-
venous technique is unique and will improve dramatically
and immediately the clinician's ability to understand,
diagnose and treat diseases of the central nervous
system.

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Brief Description of the Drawings

Figure 1 is a graphic representation of brain uptake ($\mu l/g$) of sucrose and bovine serum albumin (BSA) co-administered with bradykinin at several doses of bradykinin (μg/mouse).

Figure 2 is a graphic representation of the time course of brain uptake of sucrose after administration of a specific amount (10 μg) of a bradykinin analogue into different animals.

Figure 3 is a graphic representation of the time course of brain uptake of sucrose after the adminis-tration of a specific amount (100 μg) of a bradykinin analogue.

Figure 4a is a graphic representation of brain uptake of sucrose, displayed as percent of injected dose, following the administration of bradykinin and captopril or bradykinin analogues.

Figure 4b is a graphic representation of brain uptake of sucrose, displayed as percent of maximum uptake, following the administration of bradykinin and captopril or bradykinin analogues.

Figure 5 is a histogram which illustrates the effect of co-administered bradykinin on the antinociceptive activity of loperamide in the mouse tail flick assay.

Figure 6 is a histogram which illustrates the effect of a co-administered bradykinin analogue on the anti-nociceptive activity of loperamide in the mouse tail flick assay.

Figure 7 is a graphic representation of the effects of a dopamine analogue, diacetyldopamine (AcDA), on the motor activity of rats when administered alone, with bradykinin or with bradykinin and haloperidol (Haldol).

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Figure 8 is a graphic representation of the effects
of no treatment (control), treatment with cisplatin,
treatment with captopril and co-administered bradykinin,
and treatment with cisplatin, captopril and co-
administered bradykinin in the survival time (days) of
rats implanted with a brain tumor, with treatments
occuring at days 4 and 6 after tumor implantation.

Figure 9 is a graphic representation of the effects
of no treatment, treatment with cisplatin and treatment
with cisplatin, captopril and co-administered bradykinin
on survival time (days) of rats implanted with a brain
tumor, with treatment period lasting from day 4 to day
14.

Figure 10 is a graphic representation of the effects
of no treatment; treatment with cisplatin; treatment with
bradykinin, captopril and cisplatin; and treatment with a
bradykinin analogue and cisplatin on survival time (days)
of rats implanted with a brain tumor.

Figure 11 is a graphic representation of the brain
uptake of an imaging agent intravenously injected 3
minutes after either saline or one of three different
amounts of bradykinin analogue was intravenously injected
into rats.

Figure 12 is a histogram which illustrates the
effect of a bradykinin analogue co-administered with an
imaging agent on the uptake of the imaging agent into the
brain at various times after injection.

Figure 13 is a graphic representation of the effects
of a dopamine receptor antagonist (domperidone) co-
administered with a bradykinin analogue on apomorphine-
induced motor activity of rats.

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Figure 14 is a graphic representation of the effects of an angiotensin II analogue co-administered with a bradykinin analogue on drinking behavior in rats.

Figure 15 is a histogram which illustrates the effects of an angiotensin II analogue and inhibitor co-administered with a bradykinin analogue on drinking behavior in rats.

Detailed Description of the Invention

This invention relates to a method for increasing the permeability of the blood-brain barrier of a host to a molecule present in the host's bloodstream. The increase in permeability of the blood-brain barrier is accomplished by intravenously co-administering a bradykinin agonist of blood-brain barrier permeability to the host. By the phrase "bradykinin agonist of blood-brain barrier permeability" is meant a compound that elicits an increase in blood-brain barrier permeability in the manner of bradykinin.

The host can be any animal which possesses a central nervous system (i.e., a brain). Examples of hosts include mammals, such as humans, domestic animals (e.g, dog, cat, cow or horse) and animals intended for experimental purposes (e.g., mice, rats, rabbits).

The molecule in the host's bloodstream can be exogenous to the host. For example, it can be a neuro-pharmaceutical agent which has a therapeutic or pro-phylactic effect on a neurological disorder. Examples of neurological disorders include cancer (e.g., brain tumors), Autoimmune Deficiency Syndrome (AIDS), epilepsy,

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Parkinson's disease, multiple sclerosis, neurodegener-
ative disease, trauma, depression, Alzheimer's disease,
migraine, pain, or a seizure disorder.

Classes of neuropharmaceutical agents which can be
used in this invention include antimicrobial agents,
antiparasitic agents, agents that act on the autonomic
nervous system including adrenergic agents and
catecholaminergic agents, anticonvulsants, nucleotide
analogues, antineoplastic agents, anti-trauma agents,
excitatory amino acids and inhibitors and other classes
of agents used to treat or prevent a neurological
disorder. Examples of antibiotics include amphotericin
B, gentamicin sulfate, pyrimethamine and penicillin. An
example of an adrenergic agent (including blockers) is
atenolol. Examples of catecholaminergic agents include
dopamine, diacetyldopamine and domperidone. Examples of
antineoplastic agents include adriamycin, methotrexate,
cyclophosphamide, etoposide, carboplatin and cisplatin.
An example of an anticonvulsant which can be used is
valproate. Examples of anti-trauma agents which can be
used include calpain inhibitors, channel blockers,
glutamate chelators and oxygen radical scavengers.
Nucleotide analogues which can be used include azido
thymidine (AZT), dideoxy Inosine (ddI) and dideoxy
Cytidine (ddC).

The molecules in the host's bloodstream can also be
diagnostic imaging or contrast agents. Examples of
diagnostic agents include substances that are labelled
with radioactivity, such as 99m-Tc glucoheptonate or
substances used in Magnetic Resonance Imaging (MRI)

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procedures, such as Gadolinium-doped chelation agents
(e.g., Gd-DTPA).

The route of administration of exogenous molecules
to the host's bloodstream can be parenterally by sub-
cutaneous, intravenous or intramuscular injection or by
absorption through a bodily tissue, such as the digestive
tract, the respiratory system or the skin. The form in
which the molecule is administered (e.g., capsule,
tablet, solution, emulsion) will depend, at least in
part, on the route by which it is administered.

The administration of the exogenous molecule to the
host's bloodstream and the intravenous injection of
bradykinin agonist of blood-brain barrier permeability
can occur simultaneously or sequentially in time. For
example, a therapeutic drug can be administered orally in
tablet form while the intravenous administration of a
bradykinin agonist of blood-brain barrier permeability is
given later (e.g. 30 minutes). This is to allow time for
the drug to be absorbed in the gastrointestinal tract and
taken up by the bloodstream before the agonist is given
to increase the permeability of the blood-brain barrier
to the drug. On the other hand, the bradykinin agonist
of blood-brain barrier permeability can be administered
before or at the same time as an intravenous injection of
a drug. Thus, the term "co-administration" is used
herein to mean that the bradykinin agonist of blood-brain
barrier permeability and the exogenous molecule will be
administered at times that will achieve significant
concentrations in the blood producing the simultaneous
effects of increasing the permeability of the blood-brain

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barrier and allowing the maximum passage of the exogenous
molecule from the blood to the interstitial fluid of the
central nervous system.

In addition, the molecule to be delivered to the
brain via the host's bloodstream can be endogenous to the
host. That is, it can be a biological product that is
naturally synthesized and produced by the host. Examples
of such biological products include sugars, such as
glucose, and small peptides, such as enkephalins and
thyroid stimulating hormone releasing factor.

Compounds are termed agonists when they increase or
elicit a physiological response by interacting with
structural moieties at particular anatomical regions of
the body. For purposes of this invention, a compound is
an agonist of blood-brain barrier permeability when it
interacts with structural moieties anatomically
associated with the blood-brain barrier to significantly
increase the permeability of the blood-brain barrier for
the molecule of interest. This effect of increased
permeability of the blood-brain barrier is believed to
operate through a tissue-associated receptor mediated
event, probably through the B_2 receptors. Other tissue-
associated receptors may similarly be involved in this
process to increase the permeability of the blood-brain
barrier, particularly when peptides or compounds other
than bradykinin or bradykinin analogues are utilized as
the agonist of blood-brain barrier permeability.
Examples of agonist compounds which initiate an increased
permeability of the blood-brain barrier to a molecule of
interest include bradykinin, bradykinin analogues, other

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peptides and other compounds that mimic the increase in permeability of the blood-brain barrier in the manner elicited by bradykinin. Since these substances apparently interact with tissue-associated receptors to produce an increase in the permeability of the blood-brain barrier to a molecule of interest, these substances can collectively be called "receptor mediated permeabilizers".

Bradykinin is a naturally occurring peptide comprised of nine amino acids having the following sequence: NH_2-Arginine-Proline-Proline-Glycine-Phenylalanine-Serine-Proline-Phenylalanine-Arginine-COOH (Lehninger, A.L., Biochemistry, p. 97 (1975)). A bradykinin analogue can be a structural derivative of bradykinin. Such analogues can be compounds which are derivatives of the number and/or sequence of amino acids in the bradykinin structure mentioned above which have a similar or enhanced effect on permeability of the blood-brain barrier. Bradykinin analogues also include modifications to the bradykinin molecule effected by changing or chemically modifying the normal amino acids, modifying peptide bonds, adding C-terminal and/or N-terminal extensions, etc.

A method for preparing bradykinin analogues is Merrifield's procedure of solid-phase peptide synthesis (Merrifield, R.B., J. Am. Chem. Soc., 86: 304 (1964); Draprau, G. and Regoli, D., Methods in Enzymology, 163: 263-272 (1988)). The first step in a solid-phase synthesis of bradykinin analogues is the formation of a covalent bond between the C-terminal protected amino acid

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of the chosen peptide sequences and the solid support or
resin. The peptide chain is built up residue by residue
by repetitive cycles of deprotection, during which the
N-terminal Boc-protecting (N-tert-butoxycarbonyl) group
is removed by trifluoroacetic acid (TFA). This is
followed by neutralization with diisopropylethylamine
(DIEA) of the amino group left as a salt and coupling of
the next amino acid in the sequence. The cycle is
repeated until the sequence is completed. After its
complete assembly, the peptide is cleaved from the resin
and purified.

Compounds that mimic the bradykinin-like increase in
permeability of the blood-brain barrier can also be
synthesized as polypeptides, purified from mixtures of
naturally occurring polypeptides, be chemically modified
forms of such polypeptides or be chemically synthesized
structures that act as bradykinin agonists of blood-brain
barrier permeability. The essential characteristic of
these compounds is in their ability to elicit an increase
in blood-brain barrier permeability in a manner similar
to that of bradykinin.

An effective amount of a bradykinin agonist of
blood-brain barrier permeability is that amount which
will significantly increase the blood-brain barrier
permeability for the molecule of interest. In other
words, it will increase the permeability of the blood-
brain barrier to allow sufficient quantities of a
molecule to pass from the blood to the interstitial fluid
of the brain to exert a therapeutic or prophylactic
effect or allow diagnostic procedures. The effective

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amount will be determined on an individual basis and will be based, at least in part, on consideration of the host's size, the specific disease, the severity of symptoms to be treated, the result sought and the specific bradykinin agonist of blood-brain barrier permeability used. Thus, the effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

The increase in permeability of the blood-brain barrier in response to a bradykinin agonist relates not only to the quantity of molecules passing from the blood to the interstitial fluid of the brain, but also, to the type of molecule to be passed from the blood to the interstitial fluid of the brain. The effect of bradykinin agonists of blood-brain barrier permeability is to preferentially increase the passage of small molecular weight substances. For example, the data listed in Example VII demonstrate that the permeability of the blood-brain barrier of mice to a 40,000 molecular weight molecule was not substantially affected while the permeability of molecules with molecular weights of 1,000 or less was statistically significantly increased. Thus, while the permeability of small molecular weight substances is increased, the exclusion of the blood-brain barrier to substances of significantly higher molecular weight is substantially maintained. (See Example VII for further detail.)

Those skilled in the art will understand that mixtures of bradykinin analogues can also be administered as agonists of blood-brain barrier permeability. For

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example, bradykinin plus a bradykinin analogue, such as
A-7, could be administered together. In like manner, A-7
could be administered with one or more other bradykinin
analogues or with bombesin or VIP or with other agonists
of blood-brain barrier permeability.

It will also be understood by those skilled in the
art that the agonists of blood-brain barrier permeability
should be non-toxic to the host. Agonists are non-toxic
if a therapeutically effective amount does not result in
death to the host.

In a specific application of the invention, a
patient afflicted with toxoplasmosis encephalitis can be
treated by administering an effective amount of an
antiparasitic agent, such as clindamycin or gentamycin,
and co-administering an angonist of blood-brain barrier
permeability. The co-administration of the agonist of
blood-brain barrier permeability allows passage of the
antiparasitic agent through the blood-brain barrier to
treat the parasitic infection.

The invention is further illustrated by the
following specific examples.

Example I. Effect of Intravenous Co-Administration
Bradykinin on Brain Tissue Uptake of Sucrose and Bovine
Serum Albumin (BSA).

Female Balb/C mice weighing approximately 20g were
employed. All solutions were prepared in sterile PBS.
Intravenous injections (100 μl) were performed in the
tail vein. ^{3}H-sucrose (10^6 cpm) was injected as a bolus
without or with bradykinin as stated. Mice were killed
10 minutes after injection. Blood was collected in

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heparinized tubes and a 100 μl aliquot was counted in a liquid scintillation counter after addition of 1 ml of 1% sodium dodecyl sulfate (SDS) to ensure solubilization of proteins, 300 μl 37% hydrogen peroxide as a bleaching agent, and 15 ml Aquasol-2 (Dupont). The brain was removed, homogenized in 5 ml H_2O, and 1 ml of the homogenate was aliquoted for liquid scintillation counting (after addition of 1 ml of 1% SDS and 15 ml Aquasol-2). Relative brain uptakes were calculated as the ratios of radioactivity recovered in brain over that recovered in blood, and expressed as [radioactivity in 1 μl blood] (μl/g)/[radioactivity in 1g brain]. In some experiments, ^{14}C-BSA (5 x 10^5 cpm) was co-injected together with ^3H-sucrose (10^6 cpm), without or with bradykinin, and the relative brain uptakes of ^{14}C-BSA and ^3H-sucrose were calculated after double isotope scintillation counting.

The results are displayed in Figure 1. As this Figure shows, the relative brain uptake of ^3H-sucrose 10 minutes after injection was significantly increased when bradykinin was co-administered with ^3H-sucrose at doses of 10, 30, 100 and 300 μg and reached a value of 33 μl/g. The threshold dose of bradykinin was 10 μg, while the two highest doses of 100 and 300 μg yielded almost identical increases in relative brain uptake of ^3H-sucrose. In contrast, bradykinin at doses of up to 100 μg had marginal effect on the relative brain uptake of ^{14}C-BSA, which remained between 14 and 18 μl/g. At a bradykinin dose of 300 μg, the relative brain uptake of ^{14}C-BSA increased to 23 μl/g.

Example II. Pharmacological Characterization of the

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Effect_of_Bradykinin,_Bradykinin_Anologues_and_Other
Peptides_on_the_Mouse_Blood-Brain_Barrier.

The protocol for these experiments was the same as
described in Example I, except as noted. Drugs were
administered to the mice at the concentrations listed in
Table 1. For bradykinin and bradykinin analogues, brain
levels of ^{3}H-sucrose were determined 10 minutes after
treatment. For bombesin and vasoactive intestinal
peptide, brain levels of ^{14}C- sucrose were determined 10
minutes after treatment. The brain uptake data are
expressed as mean ± s.d. and are derived from two
independent experiments except for the desArg^9-
bradykinin, bombesin and vasoactive intestinal peptide
results which were derived from one experiment for each
peptide. Four mice were used for each different drug per
experiment. The results are as follows:

Table_I

Treatment	Dose/ Mouse	N	Brain Uptake of sucrose (% Control)	% Increase over Control
None (Control)		8	100 ± 9	0
Bradykinin	300 μg	8	177 ± 24	77
[Phe^{8}Ψ(CH$_2$-NH)Arg^9]bradykinin	125 μg	8	174 ± 15	74
N-acetyl[Phe^{8}Ψ(CH$_2$-NH)Arg^9] bradykinin	100 μg	5	230 ± 26	130
desArg^9-bradykinin	300 μg	4	90 ± 15	-10
Bombesin	10 μg	4	207 ± 14	107
Vasoactive Intestinal Peptide	10 μg	4	209 ± 7	109

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Bradykinin significantly increased the brain uptake of ^{3}H-sucrose. In addition, the bradykinin analogue and agonist [Phe^{8}Ψ(CH$_2$-NH)Arg^9]bradykinin, which is believed to be specific for B$_2$ receptors, also increased brain uptake. This agonist differs from bradykinin in that the peptide bond between the 8th (Phe) and 9th (Arg) amino acids is replaced with the isosteric CH$_2$-NH bond in order to protect bradykinin from degradation. When this agonist is treated further with N-acetylation to give N-acetyl[Phe^{8}Ψ(CH$_2$-NH)Arg^9] bradykinin, the effect on brain uptake is increased to a similar extent as that obtained with bradykinin or the original analogue. This form of the analogue is also devoid of histamine-releasing activity, unlike bradykinin itself. One product of bradykinin degradation is desArg^9-bradykinin. This degradation byproduct results from the action of kininase 1 (carboxypeptidase N), a proteolytic enzyme, which removes the C-terminal arginine . This bradykinin analogue is believed to retain biological activity on B$_1$ receptors, but did not substantially affect the brain uptake of sucrose through the blood-brain barrier.

The peptides bombesin (p Glutamate-Glutamine-Arginine-Leucine-Glycine-Asparagine-Glutamine-Tryptophan-Alanine-Valine-Glycine-Histidine-Leucine-Methionine-NH$_2$) and vasoactive intestinal peptide (Histidine-Serine-Aspartate-Alanine-Valine-Phenylalanine-Threonine-Aspartate-Asparagine-Tyrosine-Threonine-Arginine-Leucine-Arginine-Lysine-Glutamine-Methionine-Alanine-Valine-Lysine-Lysine-Tryptophan-Leucine-Asparagine-Serine-Isoleucine-Leucine-

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Asparagine-NH$_2$) significantly increased the brain uptake of ^{14}C-sucrose. This indicates that these peptides are receptor mediated permeabilizers.

Example III.   Synthesis of the Boc-4-MeTyr (ΨCH$_2$NArg (Tos)-O-Resin

N-BOC-O-Methyl-L-Tyrosine N-Methoxy-N-methylamide

To 350 ml of anhydrous THF on ice was added 3.635g (37.2 mmols) of N,O-dimethylhydroxylamine hydrochloride. The mixture was stirred for 10 minutes to allow most of the N,O-dimethylhydroxylamine hydrochloride to dissolve. Then, the following ingredients were successively added to the flask: 10g (33.8 mmols) of N-Boc-O-methyl-L-tyrosine, 6.977g (33.8 mmols) of dicyclohexylcarbo-diimide, 1.96g (16.04 mmols) of 4-dimethylaminopyridine, and 6.209ml (35.64 mmols) of N,N-diisopropylethylamine. When all of the reagents had been added, the reaction flask was placed in a cold room (4°C) and stirred for 12 hours. The contents of the flask were gravity filtered using Whatman Qualitative #1 filter paper. The filtrate was concentrated by means of a rotary evaporator to viscous oil which was then redissolved in 200 mls of methylene chloride. This crude reaction mix was allowed to sit at 4°C for one hour and then filtered as before in order to remove any residual dicyclohexylurea. The filtrate was again concentrated by means of a rotary evaporator and redissolved in 50 ml of methylene chloride in preparation for column chromatography. Column chroma-tography was performed using silica (230-400 mesh, 60A)

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as the adsorbent and 50/50 ethyl acetate/hexane as the
eluant. The column used for this scale reaction was 70
cm in length and 10 cm in diameter. The product eluted
after approximately 400 ml of eluant had been run through
the column. The fractions were all checked by TLC using
Silica Gel 60 F-254 glass backed plates. The desired
product (Rf value of 0.46) was pooled and concentrated in
vacuo. Concentration afforded clear, colorless oil which
was placed under high vacuum for several hours. At the
end of this time the product remained as a semi-solid
material and with time became completely solid.

There remained 5.73 g (50.2%) of a white solid with
a mp of 58-62°C; IR (cm^{-1}, KBr) 3320, 2980, 2840, 1710,
1655, 1520, 1205, 1180; MS $\underline{mle}$ 338.4 (M+); ^{1}H (CDCl$_3$, 300
MHz) δ7.08 (d,2H, J=8.50 Hz), δ6.82 (d, 2H, J=8.50 Hz),
δ5.15 (br d, 1H, J=8.90 Hz), δ4.89 (br m, 1H), δ3.78 (s,
3H), δ3.66 (s, 3H), δ3.17 (br s, 3H), δ2.99 (d of d, 1H,
J=6.0 Hz), δ2.83 (d of d, 1H, J=6.0 Hz), δ1.39 (s, 9H);
Anal. Calcd; C, 60.35; H, 7.69; N, 8.28. Found: C,
60.58; H, 8.02; N, 8.31.

N(t-Butoxycarbonyl)-O-methoxy-L-tyrosinal:

To 150 ml of anhydrous ethyl ether was added 1.04g
(27.37) mmols of lithium aluminum hydride and the
suspension was gently refluxed for 30 minutes. Upon
cooling to 4°C, the reflux condenser was replaced by a
pressure equalizing dropping funnel containing 7.4g (21.9
mmols) of N-(t-butoxycarbonyl)-O-methyl-L-tyrosine
N-methoxy-N-methylamide dissolved in 100 ml of anhydrous
ethyl ether. The contents of the funnel were added over

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one hour. The reaction mix was allowed to react for an
additional two hours. At the end of this time a cold
solution of KHSO$_4$ (5.433 g in 230 ml of H$_2$O) was added to
the reaction vessel. The layers were separated and the
aqueous layer was extracted three times with 150 mls of
ether each time. The ether layers were combined and
worked up as follows: washed three times with 200 mls of
3N HCl; washed three times with 200 mls of saturated
sodium bicarbonate; washed three times with 200 mls of
brine; and dried over magnesium sulfate, filtered , and
concentrated in vacuo. There remained 3.78 g (61.6%) of
a white solid with a mp of 69-72°C: Rf=0.65 in 50/50
ethyl acetate/hexane; IR (cm^{-1}, KBr) 3360, 2840, 1740,
1695, 1530, 1255, 1180: MS m/e 279.3 (M$^{+)}$; ^{1}H (CDCl$_3$),
300 MHz) δ9.63 (s, 1H), δ7.08 (d, 2H, J=8.5 Hz) δ6.84 (d,
2H, J=8.5), δ5.05 (br s, 1H), δ4.40 (m, 1H), δ3.79 (s,
3H), δ3.06 (d, 2H, J=6.50), δ1.44 (s, 9H); ^{13}C NMR
(CDCl$_3$, 75.47 MHz) δ200, 158.79, 130.28, 127.69, 114.27,
61.05, 55.29, 34.70, 28.26; Anal. Calcd: C, 64.51; H,
7.52; N, 5.01. Found: C,64.60, H, 7.86; N, 4.93.

N-BOC-4MeTyr(ΨCH$_2$NH)Arg(Tos)-OH

     To a flask containing 100 ml of Methanol:Acetic Acid
(99:1) was added 4.92g (15 mmols) of N^g-Tosyl-arginine
followed by 1.15g (18 mmol) of sodium cyanoborohydride.
The reagents were stirred for 5 minutes followed by the
addition of 4.46g of N-BOC-4-Me-Tyrosinal. After 30
minutes, an additional 1.15g (18 mmols) of sodium
cyanoborohydride was added to the reaction vessel. Three
additional portions of sodium cyanoborohydride were added

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at thirty minute intervals and the reaction was allowed
to stir overnight. The reaction was worked-up by
evaporating the solvent. The residue was dissolved in
heptane and dried followed by dissolution in ether and
drying. Water (200 ml) was added to the flask and the
solid collected by filtration. TLC analysis revealed a
homogenous product with an Rf of 0.35 in CHCl3:MeOH,4:1).
NMR was consistent with the expected product.

N-BOC-4MeTyr(ΨCH₂N[Z])Arg(Tos)-OH

To 2.14 g (3.61 mmole) of the pseudodipeptide above
was added 1.65 g (19.6 mmole) of NaHCO₃ in 100 ml of 1:1
dioxane/water. Benzyl chloroformate (0.6 ml, 4 mmol) was
added and the reaction was stirred overnight. The
solvents were removed in vacuo leaving a gummy residue.
The residue was suspended in 100 ml of water and this was
acidified to pH2 with HCl and extracted three times with
ethyl acetate. The combined ethyl acetate fractions were
dried over magnesium sulfate, filtered and evaporated to
provide 2.35g (90%) of the desired material as a crude
amorphous white solid. Recrystallization from methylene
chloride/hexane provided 2.18g (83%) of the product as a
white solid.

Attachment of the Protected Pseudodipeptide to the Polystyrene Resin

The protected pseudodipeptide was attached to
hydroxymethyl resin (Polystyrene-1% Divinylbenzene, 0.7
mequiv./g) using dicyclohexylcarbodiimide and 4-dimethyl-
aminopyridine. To 1.87 g of hydroxymethyl resin (1.31
mmol) was added 2.28 g (3.28 mmol) of the protected

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pseudodipeptide, 410 mg (3.33 mmol) of 4-dimethylamino-
pyridine, and 25 ml of anhydrous dimethylformamide in a
50 ml polypropylene tube. To this was added 680 mg (3.3
mmol) of dicyclohexylcarbodiimide and the vessel was
shaken overnight at room temperature. The resin was
collected by filtration and washed successively three
times each with methylene chloride and methanol and dried
overnight in vacuo to provide 2.6g of resin. Sub-
stitution by weight gain was calculated to be 0.54
mmol/g.

Example IV. Synthesis and Purification of A-7

A-7 was prepared by solid-phase peptide synthesis by
sequentially assembling the remaining amino acids (in a
C- to N-fashion) onto the resin bound protected pseudo-
dipeptide. The peptide was synthesized on a Beckman 990
peptide synthesizer using the following program for each
coupling cycle, 1- Wash, CH_2Cl_2 (3 x 1 min); 2-Deprotect
40% TFA/CH_2Cl_2 (2 x 10 minutes); 3- Wash, CH_2Cl_2 (3 x 1
min); 4 - Wash. Isopropanol (2 x 1 min); 5 - Wash, CH_2Cl_2
(3 x 1 min); 6- Neutralize 5% DIEA/CH_2Cl_2 (3 x 2 min); 7
- Wash, CH_2Cl_2 (5 x 1 min); 8 - Couple (3 equivalents
Boc-Amino acid, 3 equivalents of BOP) 1 x 60 min; 9 -
Wash, CH_2Cl_2 (3 x 1 min); 10 - Wash, Isopropanol (2 x 1
min); 11 - Wash, CH_2Cl_2 (3 x 1 min); 12- Test for
coupling by ninhydrin. If recoupling was necessary as
judged by a positive ninhydrin test, a partial cycle
starting at step 6 to the end was done. Following
assembly of the complete protected peptide, the
N-terminal BOC group was removed by using steps 1-5 in
the cycle and the resin dried.

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The crude peptide was isolated from the resin by treatment of the protected peptide-resin with anhydrous HF containing 10% anisole for 1 hour at 0°C. The HF was removed in vacuo and the crude resin/peptide was washed with ether three times. The peptide was extracted with 10% acetic acid and lyophilized to yield a crude peptide.

The peptide was partially purified by HPLC using a 0.1% TFA/acetonitrile gradient (10-40% over 30 minutes) on a C_{18} reverse phase support. The peptide was further purified using isocratic 15% acetonitrile in 0.1% TFA as an eluant on a C_{18} support. The fractions from the main peak were combined to provide purified A-7 which appeared homogeneous by TLC, Electrophoresis, and HPLC. FAB/MS analysis yielded the expected MW of 1098. Amino acid analysis often 6N HCl hydrolysis (24 h @ 110°C) gave the following composition: (Ser(1)0.89, Pro (2)2.00, Gly (1)0.97, Arg (1)1.03, Thi(1)0.73g 4Me-Tyr-(ΨCH_2NH)-Arg(1), detected by an alternate method, was partially destroyed in hydrolysis.

Example V. Time Course of Blood-Brain Barrier Opening in Mice and Rats.

Female Balb/C mice weighing approximately 20 g were use. All solutions were prepared in sterile phosphate-buffered saline. Intravenous injections of 100 µl of the bradykinin analogue and agonist [Hyp^3, Thi^5,4-Me-$Tyr^8\Psi(CH_2NH)Arg^9$]bradykinin (hereinafter designated as A-7) were given in the tail vein at time = 0. This agonist differs from bradykinin in that the 3rd (Pro)

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amino acid has been replaced by hydroxyproline, the 5th
(Phe) amino acid has been replaced by thienylalanine, the
8th (Tyr) amino acid has been substituted with a methyl
group at the 4 position and the peptide bond between the
8th (4-Me-Tyr) and 9th (Arg) amino acids is replaced with
the isosteric CH_2-NH bond. At various specified times
following the injection of A-7 an injection of
^{14}C-sucrose (3 X 10^6 dpm) was also made in the tail vein.
Two, five or ten minutes later, the mice were killed.
Blood was collected in heparinized tubes and centrifuged
to separate plasma from the resulting pellet. A 100 μl
aliquot of plasma was counted by liquid scintillation
counting after the addition of 15 ml of Aquasol-2
(Dupont). The brain was removed and homogenized in 2.5
ml of water and 2.5 ml of 1% SDS. One ml of the
homogenate was aliquoted and added to 15 ml of Aquasol-2
for counting. Brain uptake of sucrose was calculated and
expressed as percent of injected dose X 100.

The results are displayed in Figure 2. As seen in
this Figure, the uptake of ^{14}C-sucrose at 10 and 20
minutes was significantly higher in the presence of A-7.
The blood-brain barrier remained permeable to sucrose for
at least 20 minutes following injection of A-7 but did
not remain open after 40 minutes. Each data point repre-
sents the mean ± s.d. from 8 mice.

The time course of blood-brain barrier opening in
rats is shown in Figure 3. The methodology is
essentially the same as in the above description except
that female Harlan Sprague-Dawley rats (150-200 g) were
used and 6 X 10^6 dpm of ^{14}C-sucrose were used. The

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blood-brain barrier remains open to sucrose for 20 minutes in rats following A-7 injection. Each data point represents the mean ± s.d. from 6 rats.

In some experiments whole blood samples and intact brain tissue were combusted in a Packard Model 307 Oxidizer and the radioactivity collected and counted in a liquid scintillation counter. The results obtained by either homogenization or combustion of samples were equivalent.

Example VI. Dose Response Relationship of Bradykinin and Analogues. Sucrose Uptake Studies.

The methodology for these experiments is similar to the above two time course studies with the exception that all mice were killed 10 minutes after receiving a single tail vein injection of ^{14}C-sucrose simultaneously with bradykinin (BK) or bradykinin analogue. Figure 4a shows the amount of ^{14}C-sucrose uptake expressed as 100 X percent of injected dose over various concentrations of bradykinin andbrandykinin analogues after 10 minutes. Figure 4b represents the percent maximum response (sucrose uptake) over the range of doses of bradykinin or bradykinin analogue shown. The bradykinin analogue and agonist ([Thi^5,Phe^{8}Ψ(CH$_2$-NH) Arg^9]bradykinin) (A-4) and, particularly, [Hyp^3,The^5,4-Me-Tyr^{8}Ψ(CH$_2$NH)Arg^9]bradykinin (A-7) are more potent than the combination of bradykinin and captopril (BK+Cap). The number of mice per data point ranged from 12 to 16.

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Example VII. Uptake of Substances of Different Molecular
Weights into the Brain of Mice When Co-administered with
A-7.

        The methodology of these experiments is similar to
that of the above two examples. Specific radioactively
labeled molecules of different molecular weight and
structure, either with saline or with 10 μg of A-7, were
intravenously injected into mice via the tail vein. The
animals were sacrificed 10 minutes after the coinjection
and the radioactivity present in brain was measured as
described previously for ^{14}C sucrose. The results of
these studies are shown in Table I.

TABLE I
Uptake of Radiolabelled Substances into the Brain

Molecule	Mol. Weight	100 X % Injected Dose	
		Control	+ 10μg A-7
^{14}C-Sucrose	342	5.9±1.4	16.5±4.9
^{3}H-Inulin	5,000	0.075±0.018	0.110±0.016
^{3}H-RNase	12,600	0.123±0.039	0.117±0.030
^{3}H-Myoglobin	14,000	0.092±0.022	0.073±0.014
^{3}H-Carbonic Anhydrase	30,000	0.090±0.013	0.106±0.015
^{3}H-Ovalbumin	46,000	0.079±0.016	0.093±0.017
^{3}H-Bovine Serum Albumin	68,000	0.333±0.098	0.209±0.056

Data are presented as mean ± s.d. for 7 to 15 mice in each group.

        It appears that substances with greater molecular
weights do not readily cross the blood-brain barrier when

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coinjected with A-7. This suggests that the blood-brain
barrier permeabilizer characteristics of A-7 are
restricted to lower molecular weight substances.

Example VIII. The Effect of Bradykinin and Analogue on
the Brain Uptake and Antinociceptive Effect of
Loperamide. Tail Flick Assay.

Study A: Bradykinin

Female Balb/C mice weighing approximately 20g were
used. The tail flick assay was performed using a Tail
Flick Apparatus model TF6 (Emdie Instruments, Maidens,
VA). The intensity of the radiant heat was adjusted
daily to yield a reaction time between 2 and 3.5 seconds
in naive untreated mice. The cutoff time was set at 7.5
seconds. The tail withdrawal reaction time of mice was
measured three times at 10 second intervals immediately
prior to intravenous injections. These three values were
averaged and taken as basal value (V_0). Another three
measurements were taken 10 minutes after intravenous
injections and averaged (V_{10}). In some experiments, the
opiate receptor antagonist naloxone (NAL) was adminis-
tered intraperitoneally 15 minutes (100 μl in sterile
PBS, 10 mg/kg) prior to intravenous administration of
bradykinin and loperamide (BK+LOP). The results were
expressed as percent antinociceptive response according
to the formula: 100 x (V_{10} - V_0)/(7.5 - V_0).

Figure 5 illustrates the effectiveness of bradykinin
on the brain uptake of the opiate receptor agonist
loperamide (LOP) by demonstration of antinociceptive

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effects. While loperamide injected intravenously at a
dose of 25 μg per mouse had no activity, a complete
antinociceptive response was observed when bradykinin (30
μg) was co-injected with the opiate, with the latency of
tail withdrawal reaching the cut-off limit of 7.5 seconds
in all mice. Pretreatment of mice with naloxone (10
mg/kg) completely antagonized this antinociceptive
activity.

Study B: [Hyp^3,Thi^5,4-Me-Tyr^8Ψ(CH$_2$NH)Arg^9]bradykinin

Female Balb/C mice weighing approximately 20 g were
used. The tail flick assay was performed using a Tail
Flick Apparatus model TF6 (Emdie Instruments, Maidens,
VA). The intensity of the heat source was adjusted daily
to yield a reaction time between 2.0 and 3.2 seconds in
naive untreated mice. The maximum time of exposure to
the heat source allowed was 7.5 seconds. The tail
withdrawal reaction time of mice was measured 4 times at
10 second intervals immediately prior to intravenous
injections via the tail vein. The last three values were
averaged and taken as the baseline value (V_0). Another
set of measurements was taken at the following intervals
after tail vein injection: immediately, 5 min, 10 min,
15 min, 30 min, and 60 min. The last three values for
each of these time points (V) was averaged. In some
experiments the opiate receptor antagonist naloxone (10
mg/kg; 100 μl in saline) was administered intra-
peritoneally 15 minutes prior to administration of A-7
(0.1 μg) and loperamide (25 μg). The results were
expressed as percent antinociceptive response according
to the formula: 100 X $(V-V_0)/(7.5-V_0)$.

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Figure 6 illustrates the ability of A-7 to enhance the permeability of the blood-brain barrier to loperamide as evidenced by the increase in % antinociceptive response. Each point represents pooled data from 2 experiments with 4 mice (total of 8 mice) 30 minutes after injection of loperamide, A-7, and A-7 and loperamide with or without naloxone pretreatment. A complete antinociceptive response was obtained when A-7 was coinjected with loperamide. The effect was completely antagonized by pretreatment with naloxone.

Example IX. The Effect of the Dopaminergic Agent Diacetyldopamine When Co-Administered with Bradykinin on the Locomotor Activity of Rats.

Five Sprague-Dawley rats (125-150g) were habituated to activity cages two days prior to the experiment. The activity cages were standard size rat cages with two photocell beams running across the floor of each cage. Any movement of the rat through either of the beams was recorded by a computer. Locomotor activity was measured as the number of beam breaks in a sequence of 10-minute intervals over two hours.

On test days, all rats were pretreated with the monoamine oxidase inhibitor nialamide (100 mg/kg i.p.). This was done to prevent the enzymatic degradation of diacetyldopamine (AcDA) by monoamine oxidases. They were then placed in the activity cages to habituate. Two hours later, rats were briefly removed from the cages and injected intravenously via tail vein injection with either AcDA (2mg) or AcDA (2mg) + bradykinin (BK) (1mg).

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The rats were immediately returned to the activity cages
and locomotor activity was recorded over 10 minute
intervals for the next two hours.

The results are displayed in Figure 7. As this
Figure shows, those rats receiving AcDA alone showed no
increase in locomotor activity during the two hour test
session. However, when AcDA + bradykinin were co-
injected, there was a significant increase in the loco-
motor behavior of the rats. These data suggest that
bradykinin had enabled AcDA to enter the brain resulting
in stimulation of central nervous system dopamine
receptors.

In order to verify that the motor increase seen was
due to direct stimulation of dopamine receptors, one
group of rats received the dopamine antagonist halo-
peridol in addition to AcDA + bradykinin. The halo-
peridol (0.2 mg/kg i.p.) was administered 15 minutes
prior to the AcDA/bradykinin injection. As can be seen
in the figure, the increased motor activity was com-
pletely abolished with the haloperidol pretreatment.

Example X.   The Effect of a Dopaminergic Antagonist When
Co-administered with A-7 on the Locomotor Activity of
Rats.

Domperidone is a dopamine receptor antagonist used
clinically as an anti-emetic by virtue of its action at
the area postrema outside the blood-brain barrier.
Reports in the literature demonstrated that domperidone
does not cross the blood-brain barrier but when given as
an injection into the cerebral ventricles, it effectively

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blocks binding of dopaminergic compounds, such as apomorphine, to dopamine receptors. A pertinent test is whether domperidone can antagonize a dopamine receptor agonist-induced increase in motor activity when co-administered with A-7 but is ineffective when administered without A-7.

Sprague-Dawley rats (125-150g) were habituated for two days to activity cages. The activity cages were standard size rat cages with two photocell beams running across the floor of each cage. Any movement of the rat through either of the beams was recorded by a computer. Locomotor activity was measured as the number of beam breaks in a sequence of 10-minute intervals over a two hour period.

The rats were given a coinjection of 10μg A-7 and 300μg domperidone, or the domperidone alone, one hour before a subcutaneous injection of apomorphine (0.75mg/kg), which is a dopamine agonist. The motor activity of the rats was measured in activity cages over 10-minute intervals for up to 2 hours post-apomorphine injection.

The results of this experiment with 3 rats in each treatment group are shown in Figure 8. The combination of A-7 and domperidone antagonized the increase in motor activity associated with apomorphine. Domperidone alone had little, if any, effect on motor activity induced by apomorphine which readily crosses the blood-brain barrier.

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Example XI. The Effect of the Anti-Tumor Agent
Cisplatin When Co-Administered with Bradykinin and
Captopril or a Bradykinin Analogue on the Survival Time
of Rats with Brain Tumor Implants.

Male Fisher 344 rats (200-250g) were anesthetized
with ketamine HCl (100 mg/kg) and acepromazine (10
mg/kg). The animals were placed in a stereotaxic frame.
The heads of the animals were shaven and a midline
incision was made to expose the skull. A small hole was
drilled over the right sensorimotor cortex. Lastly, a
100 μl cell suspension (9L glioma cells, 2.5 x 10^6
cells/ml) was injected over 5 minutes into the right
caudate putamen of each animal and the scalp sutured. On
the days that followed, the animals received treatments
as described below. From day 4 until the end of the
study or time of death, animals were observed daily for
signs of failing health. When signs of very poor health
were observed (eye hemorrhage, loss of righting reflex),
animals were killed and perfused with paraformaldehyde
for subsequent histology to verify the existence of the
tumor.

Study A

Rats were treated on days 4 and 6 after implantation
of the tumor with 600 μg cisplatin i.v. via the tail vein
(18 rats, Cis), or 1 mg of captopril i.p. followed 15
minutes later by 1 mg bradykinin i.v. (18 rats, Cap-Bk),
or 1 mg captopril i.p. followed 15 minutes later by 1 mg
bradykinin i.v. followed immediately thereafter by 600 μg
cisplatin i.v., (18 rats, Cis/cap-bk). Another group of
rats received no treatment (17 rats, control).

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The results are shown in Figure 9, where survival time versus number of live rats is plotted. Treatment with captopril plus bradykinin had no effect on survival time. Treatment with the antineoplastic agent, cisplatin, did however significantly increase survival time and pretreatment with captopril and bradykinin before administering cisplatin significantly augmented this effect. The curves were fitted assuming they follow a Weibull distribution. The mean survival times and corresponding standard deviations are given in the following table in days:

TABLE II

Group	Mean	S.D.
Control	10.0	2.37
Cisplatin	13.1	2.90
Captopril-Bradykinin	10.0	1.84
Cisplatin/captopril-Bradykinin	16.5	5.77

When the anti-tumor agent, cisplatin, was administered alone, the survival time increased from 10.0 to 13.1 days. However, when cisplatin was co-administered with bradykinin and captopril, the survival time increased even further to 16.5 days. These data suggest that the pretreatment of bradykinin (with captopril to prevent its degradation) increases the blood-brain barrier's permeability to cisplatin and thereby increases the anti-tumor agent's effectiveness in the brain. The Cis/Cap-Bk group was statistically significantly different from the Cis group with a p value less than 0.025.

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Study B

Rats were treated daily from day 4 to day 14 after
implantation of the tumor with 200 μg cisplatin i.v. (9
rats, Cis) or 1 mg captopril i.p. followed 15 minutes
later by 1 mg bradykinin i.v. plus 200 μg cisplatin i.v.
(10 rats, Cis/Cap-Bk). Another group received captopril
and bradykinin, without cisplatin (9 rats, control).

The results are shown in Figure 10 and confirm the
earlier results in Study A which demonstrated that
pretreatment with bradykinin and captopril significantly
increase the effect of cisplatin on the survival time of
rats with brain tumor implants. The mean survivals and
corresponding standard deviations are given below in
days:

TABLE III

Group	Mean	S.D.
Control	8.1	2.05
Cisplatin	11.9	2.62
Cisplatin/Captopril-Bradykinin	19.7	2.45

The survival times of rats receiving cisplatin with
bradykinin and captopril were significantly higher than
rats receiving only cisplatin. The Cis/Cap-Bk group was
statistically different from the Cis group with a p value
less than 0.005. The increased permeability of the
blood-brain barrier caused by the administration of
bradykinin (with captopril to prevent its degradation),
apparently increased the brain uptake of cisplatin and
thereby increased its therapeutic effectiveness.

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Study C: [Hyp^3,Thi^5,4-Me-Tyr^{8}Ψ(CH$_2$NH)Arg^9]bradykinin

Male Fisher 344 rats (200-250 g) were anesthetized
with ketamine HCL (100 mg/kg) and acepromazine (10
mg/kg). The animals were placed in a stereotaxic device.
The heads of the animals were shaved and a midline
incision was made to expose the skull. A small hole was
drilled over the right sensorimotor cortex. A 100 μl
cells suspension (250,000 9L Glioma cells) was injected
over 5 minutes into the right caudate putamen of each
animal and the scalp sutured. Animals were observed
daily for signs of failing health. When signs of very
poor health were observed (eye hemorrhage or loss of
righting reflex) animals were killed and the brains
examined for presence of tumor.

On days 5 through 14, the animals received the
following tail vein intravenous treatments: no treat-
ment; cisplatin 200 μg/rat; A-7 (50 μg) and 5 minutes
later cisplatin; or captopril pretreatment followed by 1
mg bradykinin 15 minutes later and cisplatin 5 minutes
after bradykinin. The results are shown in Table IV as
median with range.

TABLE IV

Treatment Group	Median Survival (days)	No. Animals
Control	14, Range 10-16	6
Cisplatin	13, Range 9-18	9
BK & Captopril + Cisplatin	16, Range 10-21	9
A-7 + Cisplatin	20.5, Range 10-62	9

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Figure 11 illustrates the survival times of all
animals in the study. It should be noted that 2 animals
in the A-7 + cisplatin treatment group had extended
survival times, with one animal dying on day 38 and the
other sacrificed at day 62. Both animals had evidence of
tumor growth.

Example XII Effect of Bradykinin on the Brain
Delivery of Various Molecules
        The protocol for these experiments was the same as
described in Example I, except as noted. Radiolabeled
compounds (i.e., molecules) were administered intra-
venously to the mice with or without 300 μg of
bradykinin. Brain levels were determined 10 minutes
following treatment. The data are derived from 2-3
independent experiments for each compound. Four mice
were used for each different molecule per experiment.
The results were as follows:

Table V

Molecule	Molecular Wt. Daltons	Increase in Brain Uptake (μl/g)	Percent Increase of Control
^{3}H-Sucrose	342	+7.5 ± 2.5*	77 ± 24%*
^{3}H-AZT	267	+6.5 ± 2.6*	34 ± 10%*
^{14}C-N-acetyl-Amphotericin B	1,000	+2.9 ± 0.9*	24 ± 7%*
^{14}C-CD-4	40,000	+1.9 ± 1.3	19 ± 13%

* Statistically significantly higher than brain uptake
  without bradykinin.

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Bradykinin significantly increased the brain uptake of sucrose, AZT and N-acetyl-Amphotericin B. At a molecular weight of 40,000, however, the uptake of CD-4 was not significantly affected. The data indicate that bradykinin preferentially increases the blood-brain barrier permeability to small molecular weight substances.

Example XIII. ^{99m}Tc-DISIDA (N-[2,6-Diisopropyl-acetanilide]iminodiacetic acid) Uptake into the Head Region (Brain) in Rats.

Female Sprague-Dawley rats (250-300g) were anes-thetized with pentobarbital (60 mg/kg) and ketamine (100 mg/kg). The femoral veins were surgically exposed and the right vein was injected either with saline or a range of A-7 concentrations. After three minutes, a bolus of ^{99m}Tc-DISIDA was injected into the left femoral vein. The rats were immediately placed on a gamma camera and the radioactivity counted intitially at 1 minute intervals and then at 5 minute intervals for 1 hour. The head region where the brain is the primary organ was identified and the amount of radioactivity present in this region is shown in Figure 12 for each of the con-centrations of A-7 tested. The data for each con-centration are radioactivity measurements from a single rat. At very early times the A-7 enhanced the uptake of ^{99m}Tc-DISIDA into this region relative to the control animal. This experiment is representative of two similar studies.

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In another set of experiments, a single intravenous injection of A-7 was given into a femoral vein of an anesthetized rat. Two minutes later, an injection of ^{99m}Tc-DISIDA was given into the contralateral femoral vein. In control animals, no A-7 was injected (sham injection). At time intervals of 2, 10, 30, or 60 minutes after the ^{99m}Tc-DISIDA injection, the rats were sacrificed, their brains removed and counted in a gamma counter. The brain uptake of ^{99m}Tc-DISIDA was calculated and expressed as percent of injected dose per organ. The biodistribution of ^{99m}Tc-DISIDA in the whole brain of untreated and A-7 treated rats at selected times post-injection is shown in Table VI and Figure 13.

TABLE VI
Biodistribution of ^{99m}Tc-DISIDA in Brain

Time (after ^{99m}Tc-DISIDA Injection)	% Injected Dost/Brain Control	+ 10µg A-7
2 min.	0.040±0.013	0.075±0.019
5 min.	0.032±0.003	0.046±0.006
10 min.	0.022±0.005	0.028±0.003
60 min.	0.004±0.001	0.010±0.003

The data are expressed as mean ± s.d. for three animals per group.

These results demonstrate that larger amounts of ^{99m}Tc-DISIDA are found in the brain of A-7 treated rats when compared to control rats at early times post-injected of the labeled agent.

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Example XIV. The Effect of Angiotensin II When Co-administered with A-7 on the Drinking Behavior of Rats.

Angiotensin II in supra-physiological concentrations has been shown to induce drinking behavior in water satiated rats. This behavior has been suggested to occur as a result of stimulation of angiotensin II receptors within areas of the brain not associated with the cerebroventricular organs. Studies were performed to evaluate the effect of co-administration of A-7 with an angiotensin II analogue that is capable of causing drinking behavior when administered at a high dose.

Rats were given a coinjection of $10\mu g$ A-7 and either 0.1, 0.3, 3, 10 or $30\mu g$ of β-angiotensin II, or the β-angiotensin II, alone, via tail vein injection. The volume of water consumed by each rat over a 1 hour interval was measured.

The results of this study with 6 rats in each dosage group are shown in Figure 14. Co-administration of A-7 and the angiotensin II analogue caused the dose response curve to shift to the left, or toward lower doses of analogue, when compared to administration of the analogue alone.

In another study, rats were given either saline, 1 μg of β-angiotensin II, 1 μg of β-angiotensin II and 10 μg of A-7, $1\mu g$ of β-angiotensin II and 8 μg of saralasin or 1 μg pf β-angiotensin II plus 10 μg of A-7 plus 8 μg of saralasin via tail vein injection. The saralasin was given because it is a known angiotensin II receptor antagonist. Again, the volume of water consumed by each rat over a 1 hour interval was measured.

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The results of this study with 3 rats in each group are shown in Figure 15. The co-administration of A-7 and β-angiotensin II causes a significant increase in water consumption compared to the angiotensin II analogue alone or together with saralasin. When saralasin is co-administered with A-7 and the analogue, the water consumption remains within a normal range which indicates an inhibition of angiotensin II analogue-induced drinking behavior by the addition of the angiotensin II receptor antagonist.

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CLAIMS

1. A method for increasing the permeability of the blood-brain barrier of a host to a molecule present in the blood stream of the host comprising co-administering intravenously to said host an effective amount of a bradykinin agonist of blood-brain barrier permeability.

2. A method of Claim 1 wherein the host is a human being.

3. A method of Claim 2 wherein the bradykinin agonist of blood-brain barrier permeability comprises bradykinin.

4. A method of Claim 2 wherein the bradykinin agonist of blood-brain barrier permeability comprises a bradykinin analogue.

5. A method of Claim 4 wherein said bradykinin analogue has significantly increased resistance to proteolytic degradation in the blood stream of the human being relative to bradykinin.

6. A method of Claim 2 wherein the bradykinin agonist of blood-brain barrier permeability comprises compounds that mimic the increase in permeability of the blood-brain barrier in the manner of bradykinin.

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7.   A method of Claim 1 wherein the bradykinin agonist
     of blood-brain barrier permeability is selective for
     bradykinin 2 (B_2) receptors.

8.   A method of Claim 2 wherein the bradykinin agonist
     of blood-brain barrier permeability and said
     molecule are simultaneously intravenously
     administered to the host.

9.   A method of Claim 1 wherein the molecule comprises a
     diagnostic imaging agent.

10. A method of Claim 9 wherein the diagnostic imaging
     agent is radiolabelled or doped with a magnetic
     resonance imaging contrast substance.

11. A method of increasing the permeability of the
     blood-brain barrier of a host to a molecule present
     in the blood stream of the host comprising co-
     administering intravenously to said host an
     effective amount of a blood-brain barrier
     permeabilizer comprising a receptor mediated
     permeabilizer.

12. A method for increasing the permeability of the
     blood-brain barrier of a host to a neuropharma-
     ceutical agent present in the bloodstream of the
     host comprising co-administering intravenously to
     said host an effective amount of a bradykinin
     agonist of blood-brain barrier permeability.

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13. A method of Claim 12 wherein the bradykinin agonist of blood-brain barrier permeability and said neuropharmaceutical agent are simultaneously and intravenously administered to the host.

14. A method of Claim 12 wherein said neuropharmaceutical agent is cisplatin.

15. A method of Claim 12 wherein said neuropharmaceutical agent is amphatericin B.

16. A method of Claim 12 wherein said neuropharmaceutical agent is azidothymidine.

17. A method of Claim 12 wherein said neuropharmaceutical agent is clindamycin.

18. A method of Claim 12 wherein said neuropharmaceutical agent is gentamicin.

19. A method for increasing the permeability of the blood-brain barrier of a human to a therapeutic agent present in the blood stream of said human comprising intravenous co-administration to said host of an effective amount of a bradykinin agonist of blood-brain barrier permeability.

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20. A method of treating a patient afflicted with
    toxoplasmosis encephalitis by administering to said
    patient a therapeutically effective amount of an
    anti-parasitic agent and co-administering therewith
    a bradykinin agonist of blood-brain barrier
    permeability.

21. A method of Claim 20 wherein said anti-parasitic
    agent comprises gentamicin.

22. A method of Claim 20 wherein said anti-parasitic
    agent comprises clindamycin.

23. A pharmaceutical composition for intravenous
    administration to a human for the purpose of
    increasing the permeability of the blood-brain
    barrier to a molecule, comprising:
    a)   the molecule to be delivered from the blood to
         the brain;
    b)   a bradykinin agonist of the blood-brain barrier
         permeability; and
    c)   a pharmaceutically acceptable carrier.
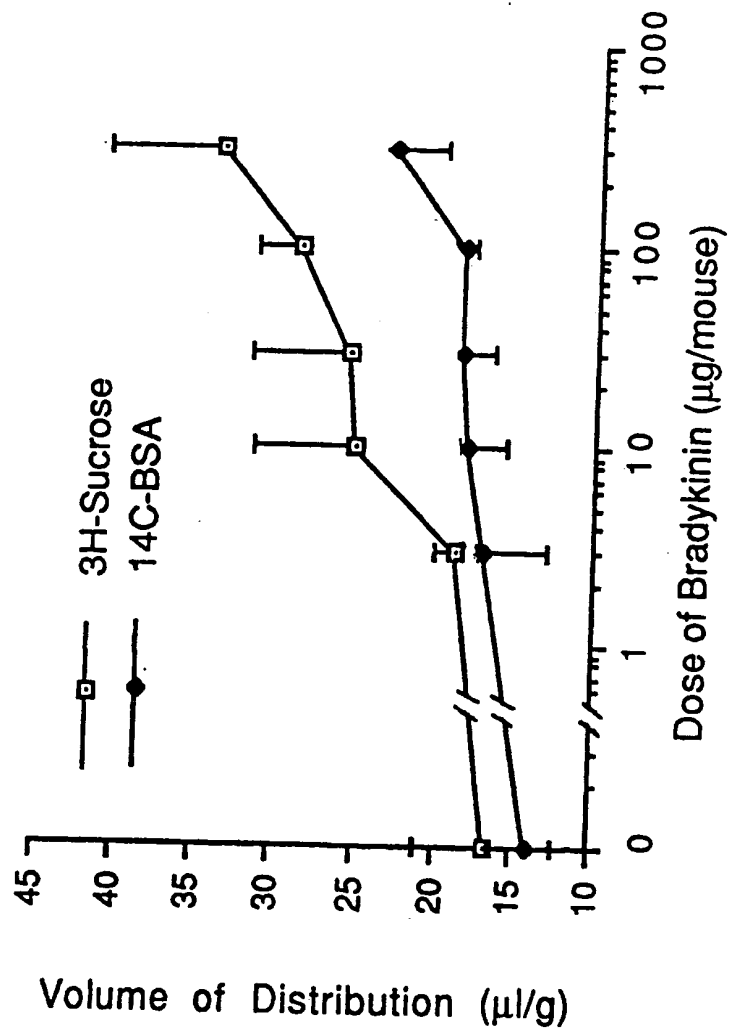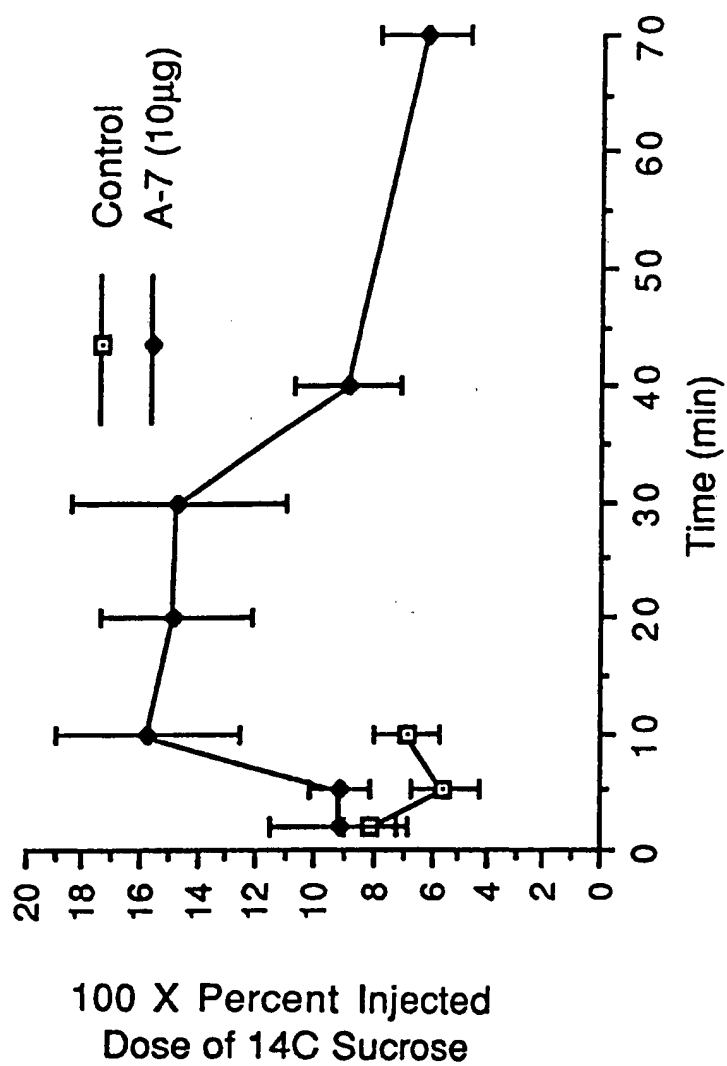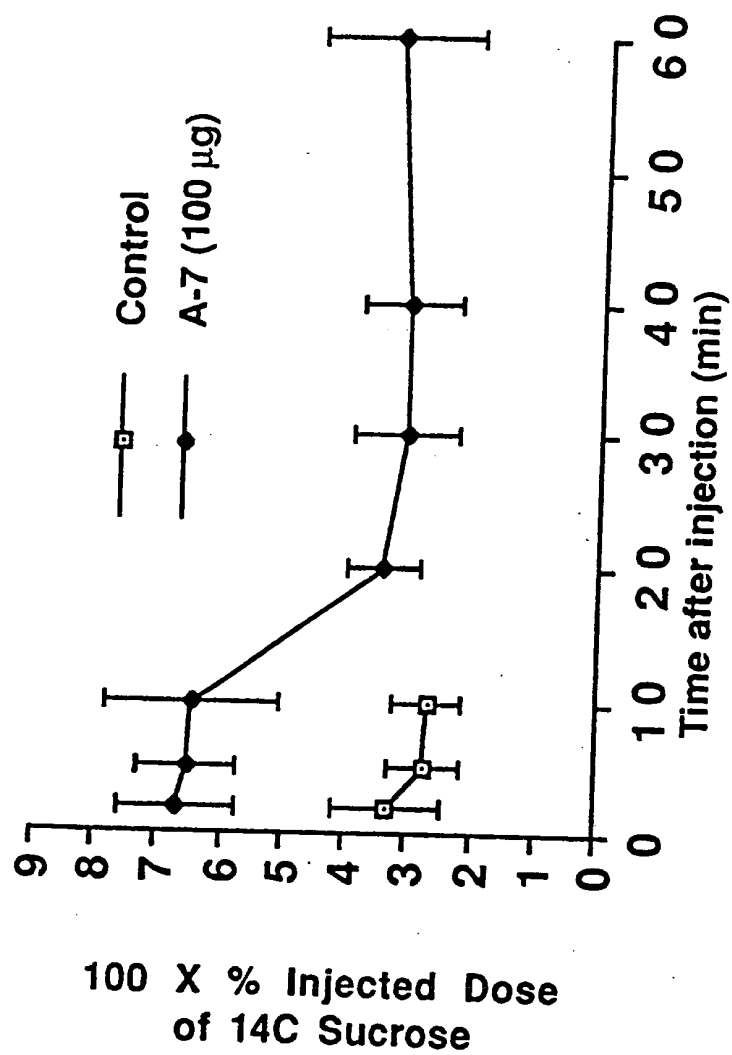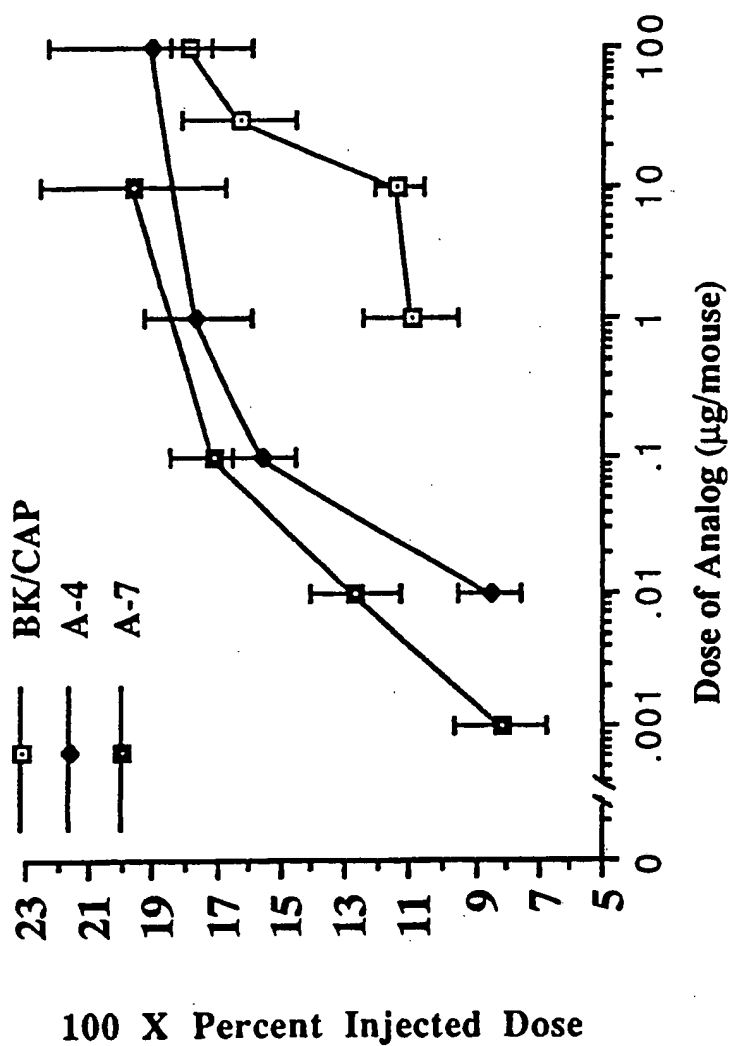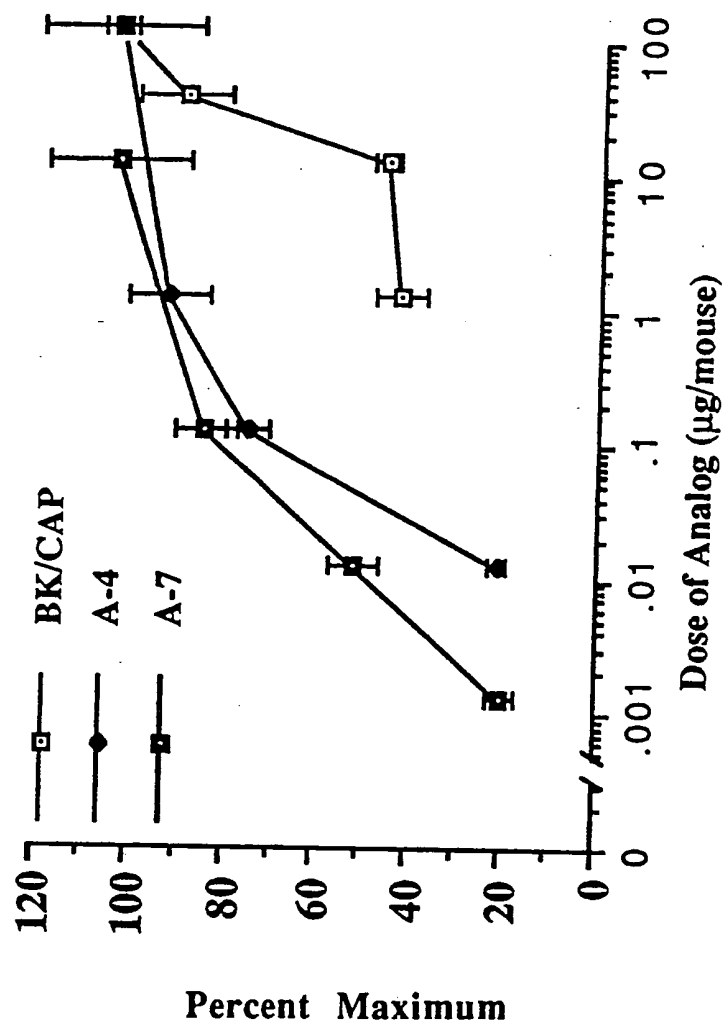
FIG. 1

FIG. 2

FIG. 3

FIG. 4a

FIG. 4b

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9

FIG. 10

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FIG.11

FIG. 12

FIG. 13

FIG. 14

Saline
β-Ang II
β-Ang II + SAR
β-Ang II + A-7
β-Ang II + A-7 + SAR

Water Consumption (mls/hr)
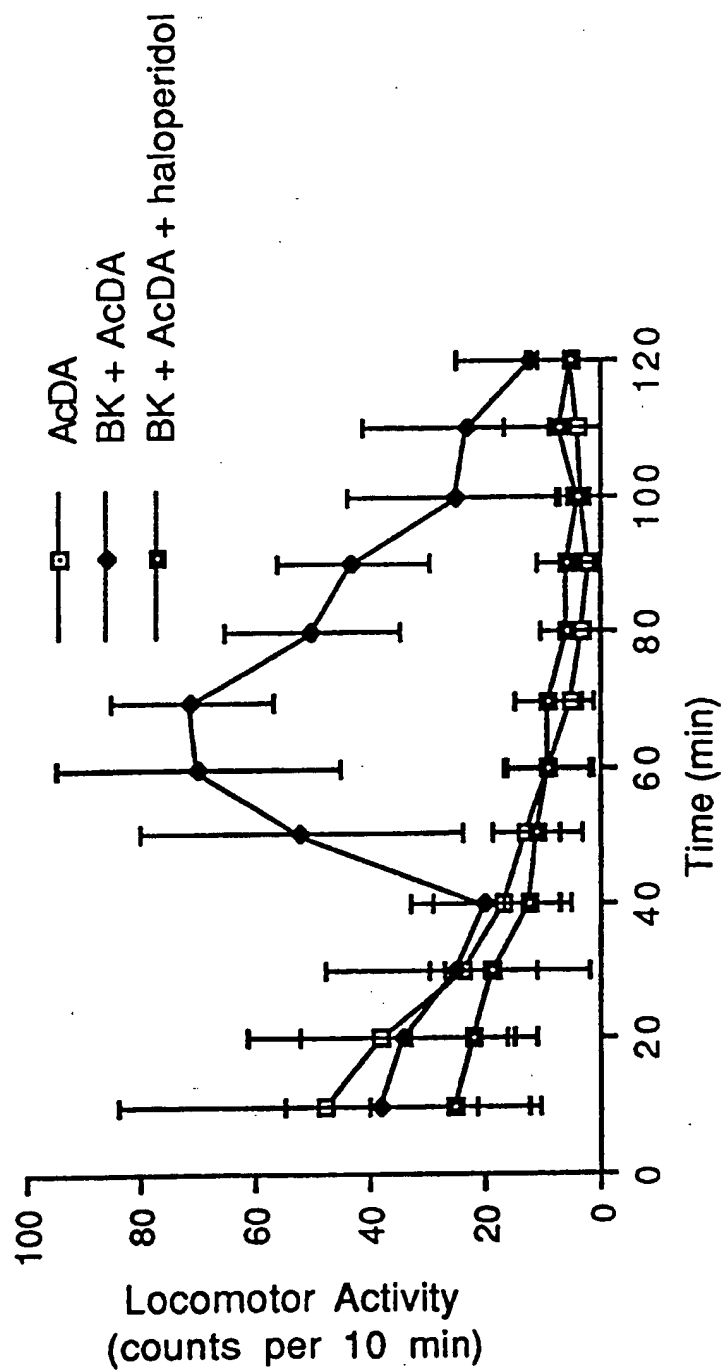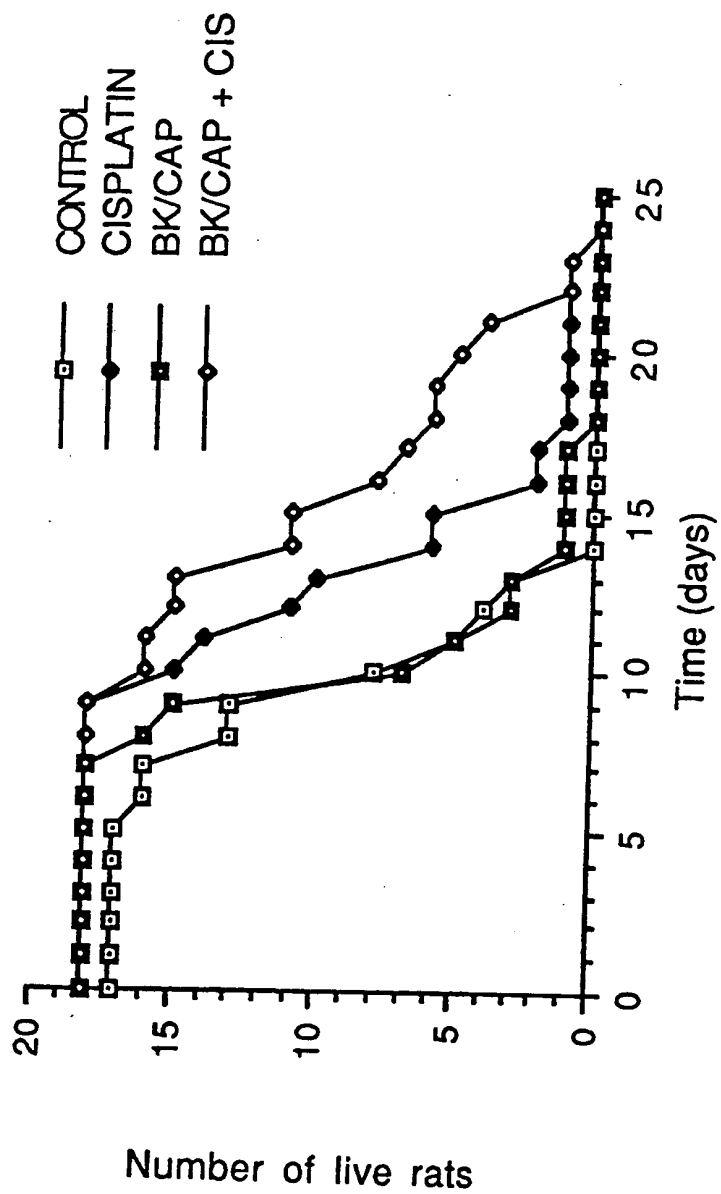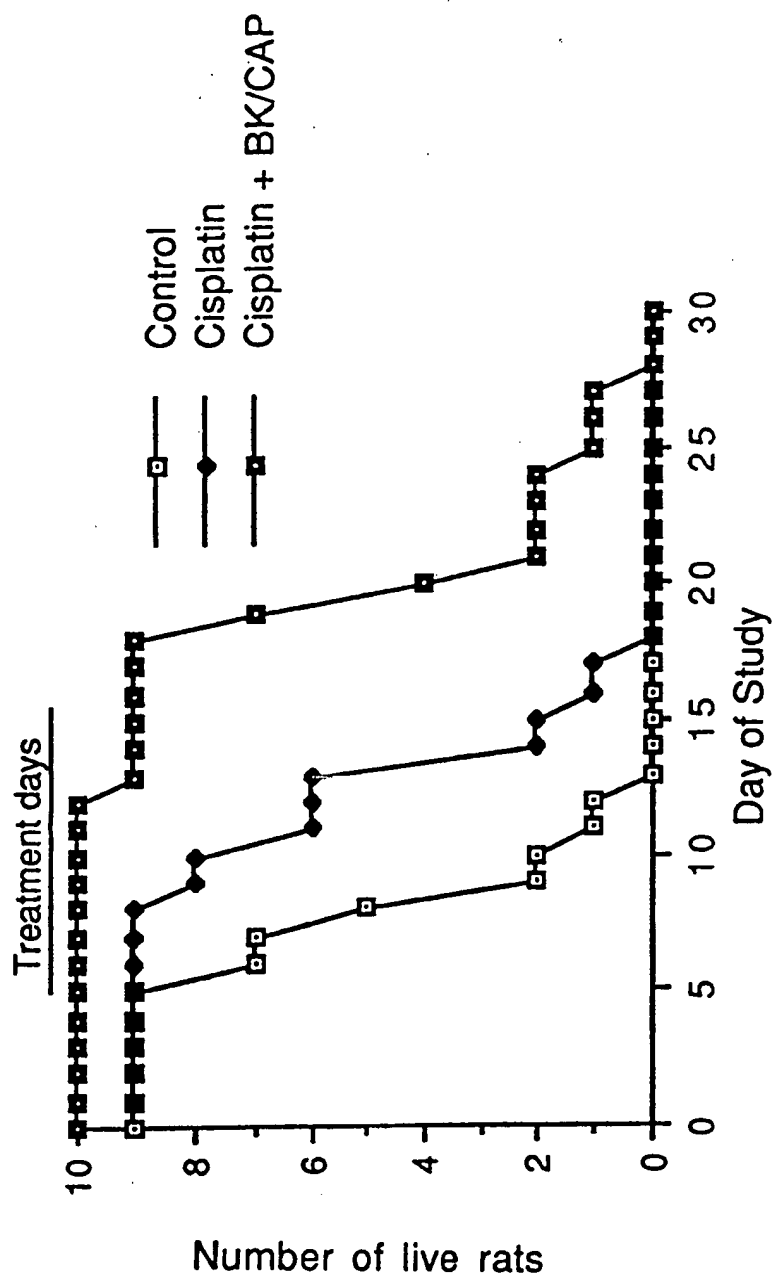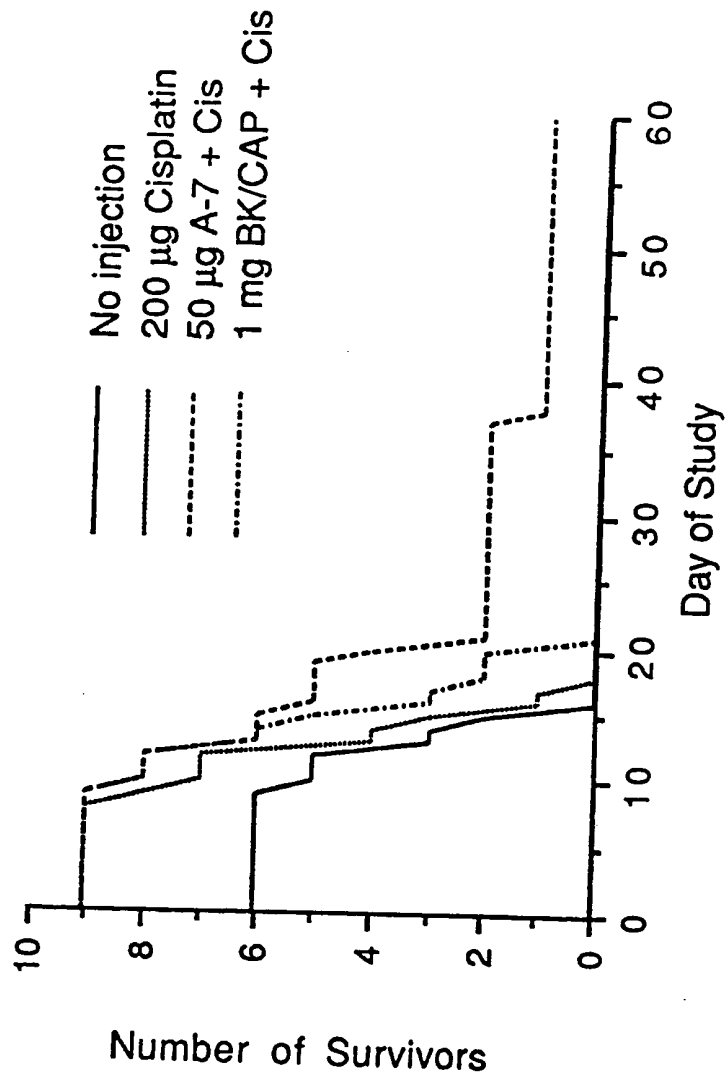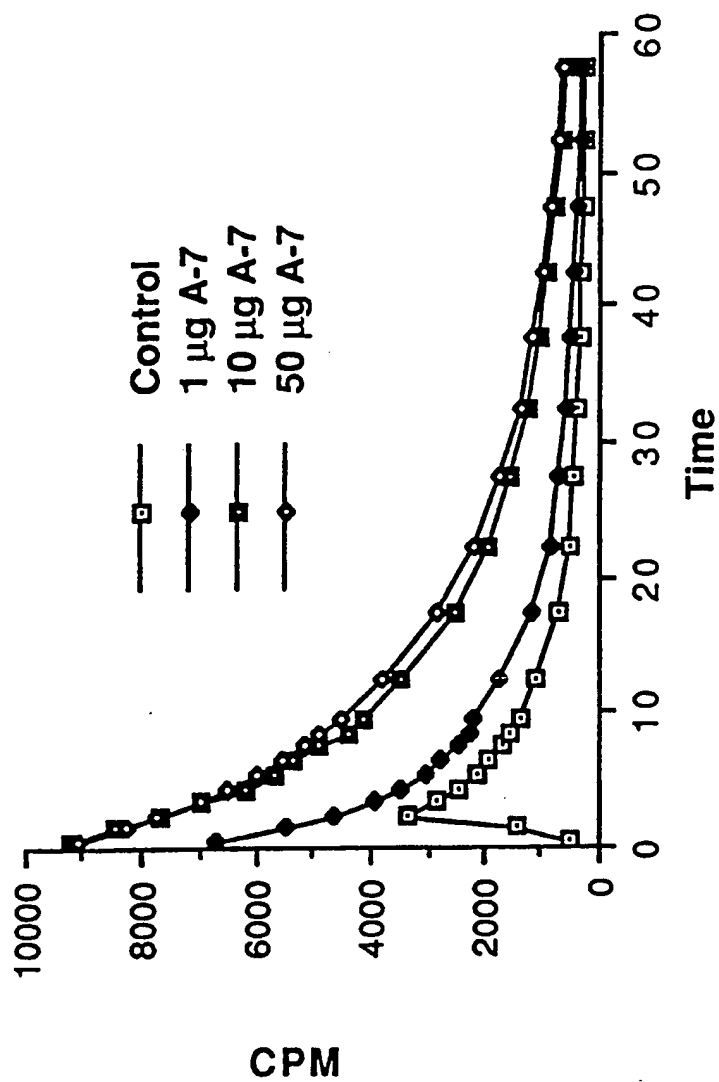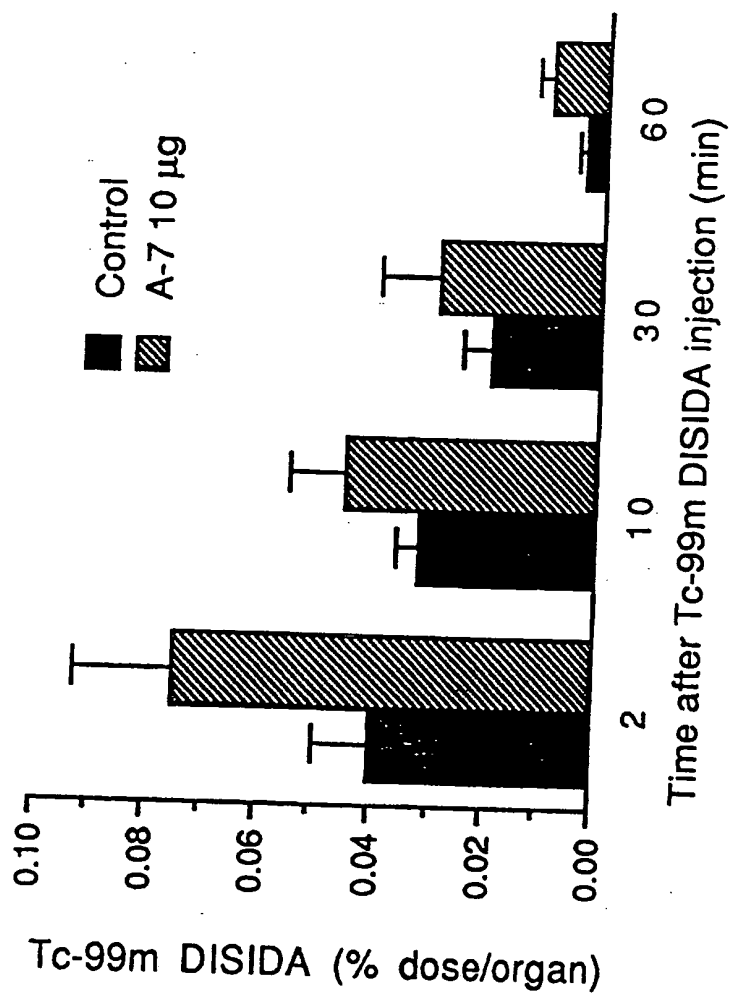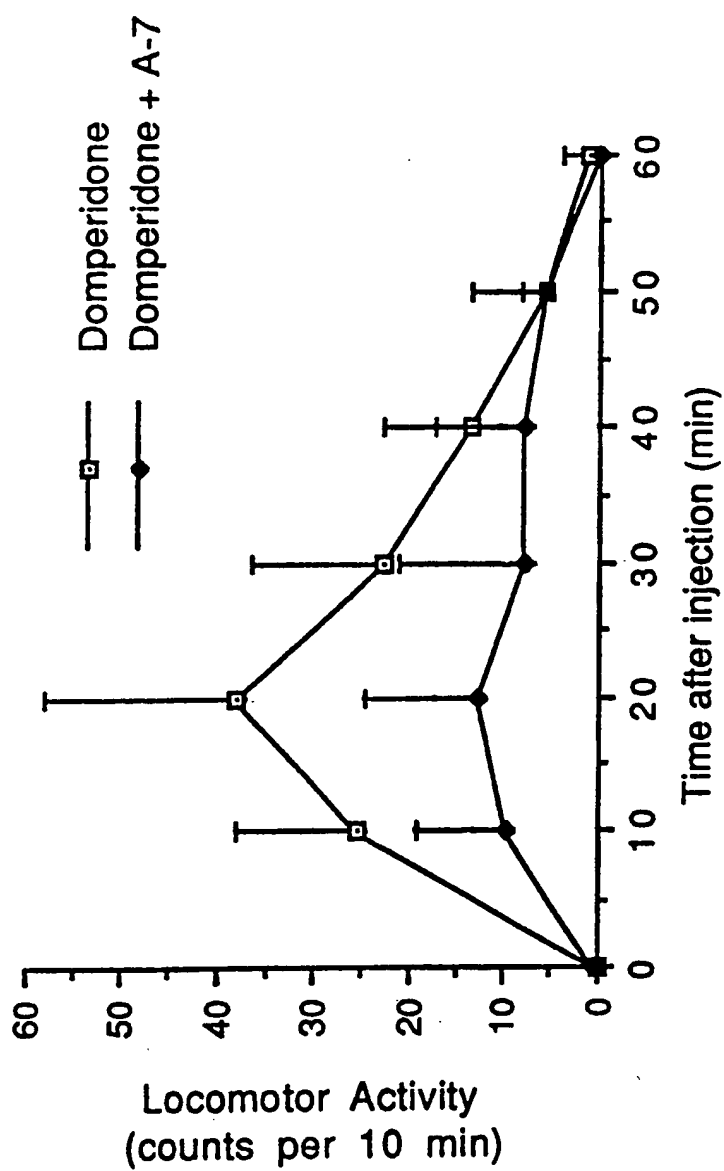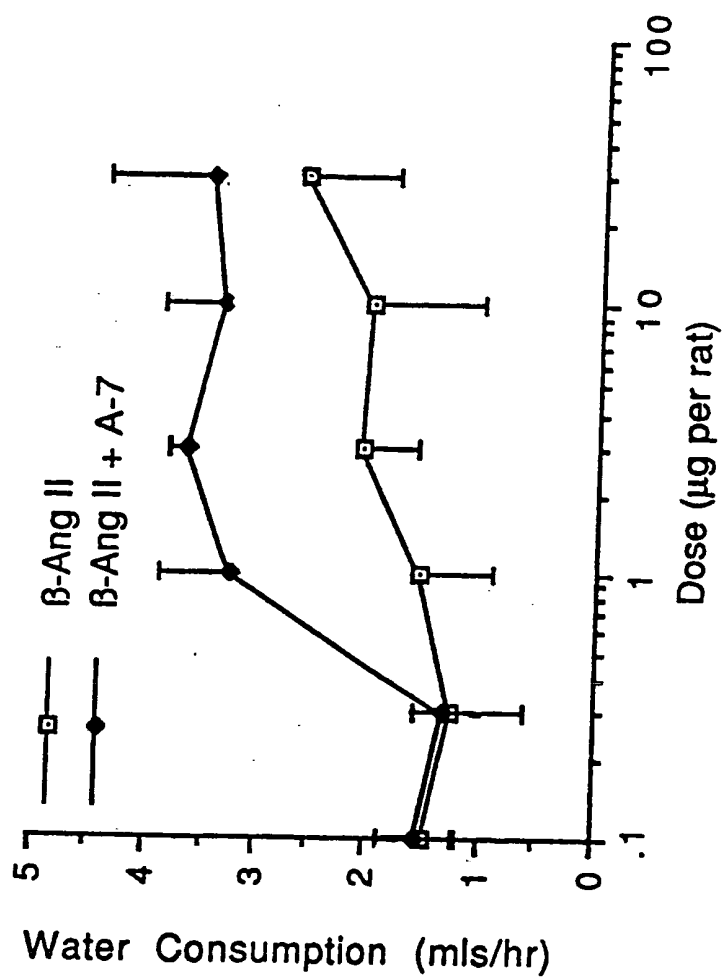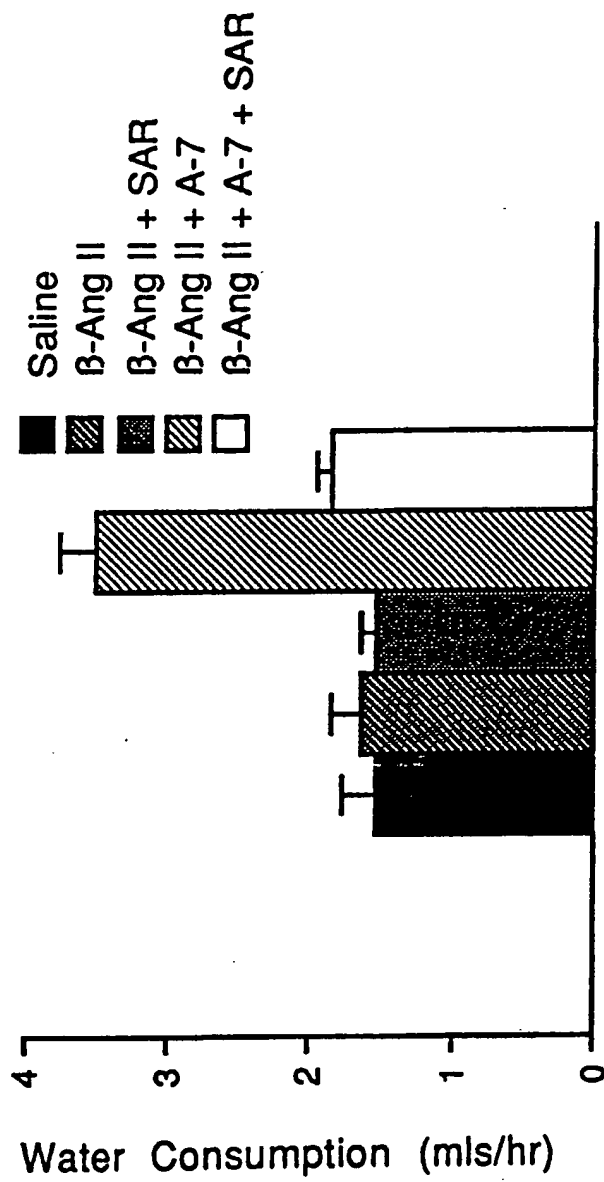
FIG. 15

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

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Int.Cl.5 ·       C 07 K  99/18    A 61 K   47/42    A 61 K   37/02  //
(A 61 K  37/02    A 61 K  33:24    A 61 K   31:71    A 61 K   31:70 )
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II. FIELDS SEARCHED

	Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	FR-M- 7 979 (SANDOZ S.A.) 8 June 1970, see page 2, lines 25-39	23

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed
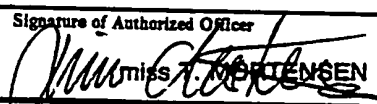
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23-08-1991	1 9. 09. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	miss A. MORTENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CO...INUED FROM THE SECOND SHEET	

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers   1 - 22   because they relate to subject matter not required to be searched by this Authority, namely:

     See PCT Rule 39.1(iv):
     methods for treatment of the human or animal body by surgery
     or therapy, as well as diagnostic methods.

2. ☐ Claim numbers   because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out. specifically:

3. ☐ Claim numbers   because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the international application

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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